ICAM-4 Binding Sites

The present invention relates to intercellular adhesion molecule-4 (ICAM-4). In particular, the invention relates to binding sites on ICAM-4, antagonists affecting ICAM-4 and uses thereof.

Intercellular adhesion molecule-4 (ICAM-4) is expressed chiefly on erythroid cells and is the glycoprotein that carries the LW blood group antigens. A study by Bailly *et al.* (1995, Eur. J. Immunol. 25: 3316-3320) showed binding of integrins LFA-1 and Mac-1 (also known as $\alpha M\beta 2$) to ICAM-4.

Another report shows that ICAM-4 binds hemopoietic (HEL) and non-hemopoietic (FLYRD18, a derivative of HT1080) cell lines and that the cellular ligands for ICAM-4 are the $\alpha_4\beta_1$ integrin and α_v integrins (most notably $\alpha_v\beta_1$ and $\alpha_v\beta_5$) respectively (Spring *et al.*, 2001, Blood 98: 458-466).

ICAM-4 possibly has a role in the formation of erythroblastic islands in the bone marrow (during erythropoiesis) and in the abnormal adhesion of red cells to activated endothelium in sickle cell disease. There is a need to understand interactions of ICAM-4 with its receptors for the development of therapies to diseases in which ICAM-4 is involved. These diseases include those involving pathology resulting from abnormal adhesion of red cells to vascular endothelium either directly, or indirectly through binding to other adhesive cells or molecules. Abnormal red cell adhesion is evident in sickle cell disease and malaria. Red cells from patients with β-thalassaemia major and β-thalassaemia intermedia also show increased adherence to endothelium and it has been suggested that this contributes to the microcirculatory disorders seen in these patients. Red cell-endothelial cell adherence has also been reported to contribute to the vascular complications found in diabetes mellitus. Red cell-endothelial cell adherence and red cell adherence to other cellular elements in the blood and wider reticuloendothelial system may also be relevant to the pathophysiology of other conditions where endothelial perturbation or vascular dysfunction occurs; such as strokes, organ transplant rejection, systemic lupus erythematosus and a range of

vasculitic and thrombotic disorders. There is preliminary evidence for the involvement of red cell adhesion via ICAM-4 in sickle cell disease and deep vein thrombosis.

According to the present invention, there is provided an epitope for binding integrins, comprising the A and G strands of domain 1 of ICAM-4 (SEQ ID NO: 1), in which the A strand (SEQ ID NO: 2) is defined by amino acid residues 17 to 27 of ICAM-4 and the G strand (SEQ ID NO: 3) is defined by amino acid residues 90 to100 of ICAM-4, or a functional homologue of the epitope.

The epitope was identified using site-directed mutagenesis of residues identified using a molecular model of ICAM-4 derived from the crystal structure of ICAM-2 (see Fig. 2). The term "ICAM-4" refers herein to the mature form of the human protein (as shown in SEQ ID NO: 1), without the N-terminal signal peptide of 30 amino acids found in precursor ICAM-4 (see Bailly *et al.*, 1994, Proc. Natl. Acad. Sci. USA91: 5306-5310). Amino acid residues are numbered with reference to this mature ICAM-4. As described in further detail below, our model predicts ICAM-4 i to have two immunoglobulin superfamily I-set domains, domain 1 being N-terminal of a membrane-anchored domain 2. According to the model, Domain 1 is an I-1 subset fold with six strands that run in order A, B, C, D, E, F and G. Hence in Domain 1 there is an ABE face and a CDFG (CFG) face. Domain 2 is an I-2 subset fold with seven strands that run in order A, B, C, C', E, F and G. Hence in Domain 2 there is an ABE and a CC'FG face. Reference to strands herein thus cover both domain 1 or domain 2 faces.

The epitope of the invention may be defined by amino acid residues F18, W19, V20 on the A strand of ICAM-4 and amino acid residues R92, A94, T95, S96 and R97 on the G strand of ICAM-4.

The epitope of the invention may be modified in that the A strand is replaced by strand F on domain 1 of ICAM-4, in which the F strand (SEQ ID NO: 4) is defined by amino acid residues 77 to 87 of ICAM-4. The epitope here may be defined by amino acid residues W77 and L80 on the F strand of ICAM-4 and amino acid residues R92, A94, T95, S96 and R97 on the G strand of ICAM-4. In the experimental section below, it is

shown integrin ligands of ICAM-4 appear to interact with the A and F strands of ICAM-4.

Mutagenesis of human ICAM-4 has revealed that modification of the above-defined single amino acids affect, for example, α_v integrin-mediated adhesion to ICAM-4, as elaborated in the experimental section below.

The epitope of the invention may be further defined by amino acid residues W66 on the E strand of domain 1 and K118 on the B strand of domain 2 of ICAM-4, in which the E strand (SEQ ID NO: 5) is defined by amino acid residues 160 to 170 of ICAM-4 and the B strand (SEQ ID NO: 6) is defined by amino acid residues 116 to 126 of ICAM-4.

The epitope may be further defined by amino acid residues N160, V161 and T162 on the E strand of ICAM-4. These residues define an N-glycosylation site which may have a role in the binding of ICAM-4 and its ligands. The glycosylation site is located on the top of the E strand (residues 160-170) of domain 2 (see Fig 5). Without an N-glycan chain formed at the N-glycosylation site, the adhesion between ICAM-4 and its ligands (for example α_v ligand) is stronger (see Fig 4 panels K and L, described below).

Integrins binding to the epitope or part thereof may be α_v integrins (for example, as found on HT1080 cells), $\alpha_4\beta1$ (also known as VLA-4; for example, as found on HEL cells and erythroblasts), or $\alpha_5\beta1$ (for example, as found on erythroblasts).

In another aspect of the invention, there is provided a footprint domain for binding integrins, comprising a first epitope as defined above and a second epitope comprising the C and F strands of domain 1 and the CE loop of domain 2 of ICAM-4, in which the C strand (SEQ ID NO: 7) is defined by amino acid residues 47 to 54 of ICAM-4, the F strand (SEQ ID NO: 4) is defined as above and the CE loop (SEQ ID NO: 8) is defined by amino acid residues 150 to 158 of ICAM-4, or a functional homologue of the footprint domain.

The footprint domain (depicted in Fig. 1 for ICAM-4) can be described as an "adhesive

footprint" for multiple integrins. The strands of ICAM-4 as defined herein arise from their position in a molecular model of ICAM-4 that is based on the crystal structure of ICAM-2 (Fig 2). Evidence is provided herewith for the involvement of the footprint domain in the interaction between ICAM-4 and multiple integrin ligands (see experimental section below).

The second epitope may be defined by amino acid residues R52 on the C strand of ICAM-4, W77 and L80 on the F strand of ICAM-4, T91, W93 and R97 on the G strand of ICAM-4, and E151 and T154 on the CE loop of ICAM-4. This second epitope has been disclosed by Hermand *et al.* (2000, J. Biol. Chem. 275: 26002-26010).

The integrins binding to the footprint domain or part thereof include α_v integrins (for example, as found on HT1080 cells), VLA-4 (for example, as found on HEL cells) and/or the β_2-family of integrins (such as Mac-1, for example, as found on leucocytes and neutrophils, and/or LFA-1), including $\alpha L\beta 2$ (for example, as found on neutrophils).

Functional homologues of the epitope or footprint domain include mammalian homologues, for example mouse homologues.

Further provided according to the invention is an antagonist of the epitope and/or the footprint domain as defined herein. For example, the antagonist may be an antibody. Antibodies have the capability to directly bind to the epitope and/or footprint domain, blocking adhesion to integrin ligands. Antibodies to ICAM-4 have been described by Bailly *et al.* (1995, Eur. J. Immunol. 25: 3316-3320) and Goel & Diamond (2002, Blood 100: 3797-3803). It is believed that those known antibodies do not bind to the epitope or footprint domain defined herein. If this is not the case, those known antibodies are excluded from this aspect of the invention.

Alternatively, an antibody may bind a separate site on ICAM-4 and alter the structural integrity of the epitope and/or footprint domain, thereby reducing affinity and/or inhibiting integrin ligand binding. It is believed that the known antibodies to ICAM described by Bailly *et al.* (1995, *supra*) and Goel & Diamond (2002, *supra*) do not alter

the structural integrity of ICAM-4 as described above. If this is not the case, those antibodies are excluded from this aspect of the invention.

Alternatively, the antagonist of the epitope and/or the footprint domain may be a compound, for example a low molecular weight compound, which binds to the epitope and/or footprint domain to reduce adhesion between ICAM-4 and its ligands.

In another aspect of the invention there is provided an antagonist of a ligand for the epitope and/or the footprint domain defined herein. The antagonist may have or consist essentially of three, four, five, six, seven, eight, nine or more amino acid residues of the A, C, F or G strands or the CE loop of ICAM-4 or a functional homologue thereof. For example, the antagonist of a ligand for the epitope and/or the footprint domain may have or consist essentially of the amino acid sequence according to SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11. The antagonist may comprise an active site having or consisting essentially of the amino acid sequence according to SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 11.

Experimental evidence (below) demonstrates inhibition of binding between ICAM-4 and various ligands (such as integrins).

Alternatively, the antagonist of a ligand for the epitope and/or the footprint domain may comprise other peptides, drugs or antibodies which bind to the ligand and thus reduce adhesion of the ligand to the epitope and/or the footprint domain

In a further aspect of the invention, there is provided a method of antagonising the epitope and/or the footprint domain, comprising the step of contacting the epitope and/or the footprint domain with the antagonist to the epitope and/or the footprint domain described herein. There is also provided a method of antagonising a ligand of the epitope and/or the footprint domain, comprising the step of contacting the ligand (or an environment such as a solution containing the ligand) with the antagonist of the ligand described herein. Our data shows that such antagonists (for example SEQ ID NOs: 9, 10 or 11) effective block binding to ICAM-4.

Another aspect of the invention is the use of the antagonist as defined herein for treating a disease, for example a disease involving ICAM-4. Furthermore, the invention covers use of an antagonist as described herein in the manufacture of a medicament for the treatment of a disease involving ICAM-4. The disease may be characterised by increased or decreased levels of ICAM-4 binding compared with ICAM-4 binding in healthy individuals.

We have found that the above epitope and footprint domain mediate adhesion to several integrins, and if this adhesion is blocked, for example, therapeutic effects may be possible in diseases such as sickle cell disease, deep vein thrombosis (DVT), malaria, strokes and more generally, vascular complications in any other condition found in mammals (heart disease, diabetes, β-thalassaemia, thrombotic complications of haematological diseases) may be possible. For example, in sickle cell disease it is thought that ICAM-4 binds sickle red cells to the endothelium. This abnormal binding may be prevented using an antagonist of ICAM-4.

In a further aspect there is provided an isolated nucleotide encoding the epitope or the footprint domain or the antagonist defined herein. For example, the isolated nucleotide encoding the epitope or the footprint domain or the antagonist may have a sequence defined within the sequence of SEQ ID NO: 12.

Embodiments of the invention will be described hereafter with reference to the accompanying figures, of which:

Figure 1 shows a molecular model of ICAM-4 depicting the entire footprint domain;

Figure 2 shows a molecular model of ICAM-4 depicting the ABE faces and the CFG faces;

Figure 3 shows a molecular model of the $\alpha_4\beta_1$ and α_V integrin binding domain of ICAM-4;

Figure 4 shows graphs A-L depicting the effect of mutating single residues of human ICAM-4Fc on the adhesion of HT1080 cells (exhibiting α_V integrin);

Figure 5 shows a molecular model of the ICAM-4 N-glycosylation site in domain 2;

Figure 6 shows a molecular model of the LFA-1 and Mac-1 binding footprint of ICAM-4;

Figure 7 is a histogram showing human ICAM-4 peptide inhibition of HEL cell binding to human ICAM-4Fc coated at 5µg/ml;

Figure 8 is a histogram showing the results of Figure 7 as a percentage of binding to human ICAM-4Fc in the absence of peptides;

Figure 9 is a histogram showing human ICAM-4 peptide inhibition of HT1080 cell binding to human ICAM-4Fc coated at 7.5µg/ml;

Figure 10 is a histogram showing the results of Figure 8 as a percentage of binding to human ICAM-4Fc in the absence of peptides;

Figure 11 is a histogram showing human ICAM-4 peptide inhibition of HEL cell binding to murine ICAM-4Fc coated at 5µg/ml;

Figure 12 is a histogram showing the results of Figure 11 as a percentage of binding to murine ICAM-4Fc in the absence of peptides;

Figure 13 is a histogram showing human ICAM-4 peptide inhibition of HT1080 cell binding to murine ICAM-4Fc coated at 5µg/ml;

Figure 14 is a histogram showing the results of Figure 13 as a percentage of binding to murine ICAM-4Fc in the absence of peptides;

Figure 15 is a histogram showing human ICAM-4 peptide inhibition of HEL cell binding to human ICAM-4Fc coated at 2.5μg/ml;

Figure 16 is a histogram showing the results of Figure 15 as a percentage of binding to ICAM-4Fc in the absence of peptides;

Figure 17 is a histogram showing human ICAM-4 peptide inhibition of HT1080 cell binding to human ICAM-4Fc coated at 5μg/ml;

Figure 18 is a histogram showing the results of Figure 17 as a percentage of binding to ICAM-4Fc in the absence of peptides;

Figure 19 is a histogram showing that ICAM-4 peptides block adhesion between erythroblasts and ICAM-4; and

Figure 20 is a histogram showing that blocking beta 2 antibody and ICAM-4 peptides inhibit adhesion between neutrophils and ICAM-4.

The figure legends in more detail are:

Figure 1 Molecular model of ICAM-4 with the entire "footprint" (The A, G and F strand of domain 1, extending down towards the CE loop of domain 2), along with residues W66 and K118 are shown in grey. Views A, B and C are rotated 120° with respect to each other.

Figure 2. Molecular model of ICAM-4 with the ABE faces shaded grey and the CFG faces are un-shaded. Views A and B are rotated 180° with respect to each other. Domain 1 is at the top of the model and is highlighted by a and domain 2 at the bottom of the model is highlighted by b.

Figure 3. The $\alpha_4\beta_1$ and α_V integrin binding footprint of ICAM-4. Views A and B are

rotated 180° with respect to each other. The mutated residues that comprise the $\alpha_4\beta_1$ and α_V integrin binding footprint in the A strand are in light grey (a), and those in the G strand are in dark grey (b). Dark grey residues in the E strand of domain 1 (W66, c) and B strand of domain 2 (K118, d) also affect $\alpha_4\beta_1$ and α_V integrin binding.

Figure 4. The effect of mutating single residues of human ICAM-4Fc on the adhesion of HT1080 cells. x-axis: wild-type and mutant human ICAM-4Fc coating concentration (μg/ml); y-axis: percentage of input cells bound. Triangles show titrations of wild-type ICAM-4Fc and diamonds show titrations of mutant ICAM-4Fc. A, F18A mutant; B, W19A mutant; C, V20T mutant; D, R92E mutant; E, A94L mutant; F, T95V mutant; G, S96A mutant; H, R97E mutant; I, W66A mutant; J, K118E mutant; K, N160A mutant; L, T162V mutant. Results shown are representative (one of several repeat experiments). Results are shown as mean (n=3) ± 1standard deviation.

Figure 5. The ICAM-4 N-glycosylation site in domain 2. Views A and B are rotated 180° with respect to each other. Residues N160 (a) and T162 (b) are highlighted in dark grey.

Figure 6. The LFA-1 and Mac-1 binding footprint of ICAM-4. Views A and B are rotated 180° with respect to each other. View A shows the Mac-1 footprint with domain 1 residues in the C, F and G strands highlighted in dark grey and domain 2 residues in the C' E loop highlighted in light grey. View B shows the LFA-1 footprint with the domain 1 residues in the F and G strands highlighted in dark grey.

Figure 7. Human ICAM-4 peptide inhibition of HEL cell binding to human ICAM-4Fc. x-axis: binding of HEL cells in the presence of assay buffer, defined peptides or EDTA; y-axis: percentage of input cells bound. a-h shows binding to human ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide (SEQ ID NO: 9); c, tRwATSRit peptide (SEQ ID NO: 10), d, rqgktlrgp peptide (SEQ ID NO: 13); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10); h,

EDTA; i, assay buffer. Human ICAM-4Fc was coated at a concentration of 5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of three independent assays.

Figure 8. Human ICAM-4 peptide inhibition of HEL cell binding to human ICAM-4Fc. x-axis: binding of HEL cells in the presence of assay buffer, defined peptides or EDTA; y-axis: input cells bound expressed as a percentage of binding to ICAM-4Fc in the absence of peptides. a-h shows binding to human ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer (100%); b, svpFWVrms peptide (SEQ ID NO: 9) (61%); c, tRwATSRit peptide (SEQ ID NO: 10) (58%); d, rqgktlrgp peptide (SEQ ID NO: 13) (107%); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13) (60%); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13) (56%); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) (35%); h, EDTA (1%); i, assay buffer (3%). Human ICAM-4Fc was coated at a concentration of 5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of three independent assays.

Figure 9. Human ICAM-4 peptide inhibition of HT1080 cell binding to human ICAM-4Fc. x-axis: binding of HT1080 cells in the presence of assay buffer, defined peptides or EDTA; y-axis: percentage of input cells bound. a-h shows binding to human ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide (SEQ ID NO: 9); c, tRwATSRit peptide (SEQ ID NO: 10), d, rqgktlrgp peptide (SEQ ID NO: 13); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10); h, EDTA; i, assay buffer. Human ICAM-4Fc was coated at a concentration of 7.5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of three independent assays.

Figure 10. Human ICAM-4 peptide inhibition of HT1080 cell binding to human ICAM-4Fc. x-axis: binding of HT1080 cells in the presence of assay buffer, defined

peptides or EDTA; y-axis: input cells bound expressed as a percentage of binding to human ICAM-4Fc in the absence of peptides. a-h shows binding to human ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide (SEQ ID NO: 9) (46%); c, tRwATSRit peptide (SEQ ID NO: 10) (60%); d, rqgktlrgp peptide (SEQ ID NO: 13) (97%); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13) (37%); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13) (44%); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) (32%); h, EDTA (6%); i, assay buffer (5%). Human ICAM-4Fc was coated at a concentration of 7.5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of three independent assays.

Figure 11. Human ICAM-4 peptide inhibition of HEL cell binding to murine ICAM-4Fc. x-axis: binding of HEL cells in the presence of assay buffer, defined peptides or EDTA; y-axis: percentage of input cells bound. a-h shows binding to murine ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide. (SEQ ID NO: 9); c, tRwATSRit peptide (SEQ ID NO: 10), d, rqgktlrgp peptide (SEQ ID NO: 13); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10); h, EDTA; i, assay buffer. Murine ICAM-4Fc was coated at a concentration of 5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 12. Human ICAM-4 peptide inhibition of HEL cell binding to murine ICAM-4Fc. x-axis: binding of HEL cells in the presence of assay buffer, defined peptides or EDTA; y-axis: input cells bound expressed as a percentage of binding to murine ICAM-4Fc in the absence of peptides. a-h shows binding to murine ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide (SEQ ID NO: 9) (67%); c, tRwATSRit peptide (SEQ ID NO: 10) (58%); d, rqgktlrgp peptide (SEQ ID NO: 13) (94%); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13) (70%); f, tRwATSRit peptide (SEQ ID NO: 10) plus

rqgktlrgp peptide (SEQ ID NO: 13) (55%); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) (47%); h, EDTA (8%); i, assay buffer (19%). Murine ICAM-4Fc was coated at a concentration of 5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 13. Human ICAM-4 peptide inhibition of HT1080 cell binding to murine ICAM-4Fc. x-axis: binding of HT1080 cells in the presence of assay buffer, defined peptides or EDTA; y-axis: percentage of input cells bound. a-h shows binding to murine ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide (SEQ ID NO: 9); c, tRwATSRit peptide (SEQ ID NO: 10), d, rqgktlrgp peptide (SEQ ID NO: 13); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10); h, EDTA; i, assay buffer. Murine ICAM-4Fc was coated at a concentration of 5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 14. Human ICAM-4 peptide inhibition of HT1080 cell binding to murine ICAM-4Fc. x-axis: binding of HT1080 cells in the presence of assay buffer, defined peptides or EDTA; y-axis: input cells bound expressed as a percentage of binding to murine ICAM-4Fc in the absence of peptides. a-h shows binding to murine ICAM-4Fc and i shows binding to human NCAMFc. a, assay buffer; b, svpFWVrms peptide (SEQ ID NO: 9) (60%); c, tRwATSRit peptide (SEQ ID NO: 10) (80%); d, rqgktlrgp peptide (SEQ ID NO: 13) (92%); e, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13) (61%); f, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13) (74%); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) (51%); h, EDTA (1%); i, assay buffer (1%). Murine ICAM-4Fc was coated at a concentration of 5μg/ml, peptides were used at 500μM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 15. Human ICAM-4 peptide inhibitions of HEL cell binding to human ICAM-4Fc. x-axis: binding of HEL cells in the presence of assay buffer, defined peptides or EDTA, y-axis: percentage of input cells bound. a -p shows binding to human ICAM-4Fc. a, assay buffer, b, assay buffer plus 2mM EDTA c svpFWVrms peptide (SEQ ID NO: 9), d, tRwATSRit peptide (SEQ ID NO: 10), e, aWssLahcl peptide (SEQ ID NO: 11), f, rqgktlrgp peptide (SEQ ID NO: 13), g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10), h, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11), i, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11), j, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13), k, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13), l, aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13), m, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrg peptide (SEQ ID NO: 13), n, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13), o, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13), p, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11). Human ICAM-4Fc was coated at a concentration of 2.5μg/ml, peptides were used at 750μM final concentration for each peptide, and each data point is the mean of two independent assays

Figure 16. Human ICAM-4 peptide inhibitions of HEL cell binding to human ICAM-4Fc. x-axis: binding of HEL cells in the presence of assay buffer, defined peptides or EDTA, y-axis: input cells bound expressed as a percentage of binding to human ICAM-4Fc in the absence of peptides. a, assay buffer; b, assay buffer plus 2mM EDTA (26%); c, svpFWVrms peptide (SEQ ID NO: 9) (64%); d, tRwATSRit peptide (SEQ ID NO: 10) (58%); e, aWssLahcl peptide (SEQ ID NO: 11) (50%); f, rqgktlrgp peptide (SEQ ID NO: 13) (105%); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) (52%); h, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11) (43%); i, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) (41%); j, svpFWVrms peptide (SEQ ID NO: 9)

plus rqgktlrgp peptide (SEQ ID NO: 13) (59%); k, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13) (55%); l, aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13) (46%); m, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrg peptide (49%); n, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13) (42%); o, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13) (40%); p, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) (42%). Human ICAM-4Fc was coated at a concentration of 2.5µg/ml, peptides were used at 750µM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 17. Human ICAM-4 peptide inhibitions of HT1080 cell binding to human ICAM-4Fc. x-axis: binding of HT1080 cells in the presence of assay buffer, defined peptides or EDTA, y-axis: percentage of input cells bound. a -p shows binding to human ICAM-4Fc. a, assay buffer, b, assay buffer plus 2mM EDTA c svpFWVrms peptide (SEQ ID NO: 9), d, tRwATSRit peptide (SEQ ID NO: 10), e, aWssLahcl peptide (SEQ ID NO: 11), f, rqgktlrgp peptide (SEQ ID NO: 13), g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10), h, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11), i, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11), j, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13), k, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13), l, aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13), m, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrg peptide , n, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13), o, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13), p, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11). Human ICAM-4Fc was coated at a concentration of 5µg/ml, peptides were used at 750µM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 18. Human ICAM-4 peptide inhibitions of HT1080 cell binding to human ICAM-4Fc. x-axis: binding of HT1080 cells in the presence of assay buffer, defined peptides or EDTA, y-axis: input cells bound expressed as a percentage of binding to human ICAM-4Fc in the absence of peptides. a, assay buffer; b, assay buffer plus 2mM EDTA (10%); c, svpFWVrms peptide (SEQ ID NO: 9) (41%); d, tRwATSRit peptide (SEQ ID NO: 10) (42%); e, aWssLahcl peptide (SEQ ID NO: 11) (71%); f, rqgktlrgp peptide (SEQ ID NO: 13) (96%); g, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) (46%); h, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11) (52%); i, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) (50%); j, svpFWVrms peptide (SEQ ID NO: 9) plus rqgktlrgp peptide (SEQ ID NO: 13) (40%); k, tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrgp peptide (SEQ ID NO: 13) (39%); l, aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13) (64%); m, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus rqgktlrg peptide (39%); n, svpFWVrms peptide (SEQ ID NO: 9) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13) (50%); o, tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) plus rqgktlrgp peptide (SEQ ID NO: 13) (48%); p, svpFWVrms peptide (SEQ ID NO: 9) plus tRwATSRit peptide (SEQ ID NO: 10) plus aWssLahcl peptide (SEQ ID NO: 11) (52%). Human ICAM-4Fc was coated at a concentration of 5µg/ml, peptides were used at 750µM final concentration for each peptide, and each data point is the mean of two independent assays.

Figure 19. ICAM-4 peptides block adhesion between erythroblasts and ICAM-4. Erythroblast adhesion to 5µg/ml ICAM-4 was performed in the presence of 5µg/ml of the beta 1 integrin activating antibody TS2/16. Peptides were used at 500µM. Results are expressed as a % of the erythroblasts bound to ICAM-4 minus those bound to NCAM in the presence of the rqgktlrgp peptide (SEQ ID NO: 13) (i.e. % control cells bound). a, svpFWVrms peptide (SEQ ID NO: 9); b, tRwATSRit peptide (SEQ ID NO: 10); c, aWssLahcl peptide (SEQ ID NO: 11); d, rqgktlrgp peptide (SEQ ID NO: 13).

Figure 20. Blocking beta 2 antibody and ICAM-4 peptides inhibit adhesion between neutrophils and ICAM-4. Neutrophil adhesion to 2.5μg/ml ICAM-4 was performed in the presence of 10μg/ml of the integrin blocking antibodies and 500μM of the peptides. Results are expressed as a % of the neutrophils bound to ICAM-4 in the absence of either antibody or peptide (i.e. % control cells bound). a, beta 1; b, beta 2; c, beta 3; d, svpFWVrms peptide (SEQ ID NO: 9); e, tRwATSRit peptide (SEQ ID NO: 10); f, aWssLahcl peptide (SEQ ID NO: 11).

Experimental

In order to elucidate the structural basis of integrin-ICAM-4 interaction, in Example 1 we analysed surface-exposed residues, by site-directed mutagenesis, using a molecular model of ICAM-4 derived from the crystal structure of ICAM-2. The model presents ICAM-4 as two Ig-like domains; domain 1 being N-terminal of the membrane anchored domain 2. Each domain has two faces (or sides); the ABE and the CC'FG faces (Fig 2). Mutagenesis of ICAM-4 has revealed that a number of single amino acid changes affect α_v integrin-mediated adhesion to ICAM-4. Peptide inhibition data confirms the mutagenesis data and provides evidence that the same footprint is relevant to ICAM-4's interaction with $\alpha_4\beta_1$. Due to the overlap of the α_v integrin and $\alpha_4\beta_1$ binding site with that of the binding site of LFA-1 and Mac-1 we predict that the peptides also inhibit any ICAM-4/LFA-1 or Mac-1 interaction. In Examples 2 and 3, we show that blocking peptides (antagonists) are capable of inhibiting erythroblast and neutrophil adhesion to ICAM-4, suggesting that such antagonists can be useful in treating diseases relating to ICAM-4 dysfunction.

Example 1

Cell adhesion assay

Cell adhesion assays were performed as described in Spring *et al.* 2001 (*supra*). Immulon-4 96 well plates (Dynex Technologies, Billingshurst, United Kingdom) were coated with 1μg/well goat-antihuman-Fc (Sigma, Poole, United Kingdom) for 24 hours at 4°C, washed three times with PBS and coated with an Fc fusion ICAM-4 protein for 18 hours at 4°C before blocking with 0.4% BSA PBS for 2 hours at 22°C. Cells were

labelled with 10μg/ml 2',7'-bis-(2-carboxyethyl)-5-(and-6-) carboxyfluorescein acetoxymethyl ester in assay buffer (IMEM, 2mM EGTA, 10μg/ml human ivIgG) for 15 minutes at 37°C. HT1080 cells were activated with 80μM phorbol myristate acetate prior to both cells being washed with assay buffer containing 2mM Mn^{2+}. Cells, at $5x10^4$ cells per well, were added to the ICAM-4Fc-coated plates for 30 minutes at 37°C, prior to being given repeated washes in assay buffer and read on a fluorescence microplate reader (excitation 485nm, emission 530nm). The percentage of bound cells was calculated after each wash. Peptide inhibition was performed by incubating the cells with 500μM peptide at 0°C for 15 minutes ahead of their addition, still in the presence of 500μM peptide to the ICAM-4Fc coated plates. In peptide inhibition studies the appropriate ICAM-4Fc coating concentration for each cell line was pre-determined by titration of ICAM-4Fc and the lowest concentration at which maximal binding was achieved was used to coat the plates

Preparation of ICAM-4Fc fusion proteins

Point mutations were inserted into ICAM-4 in pIg vector (see Simmons DL, 1993, Cloning cell surface molecules by transient expression in mammalian cells, In: Hartley DA, ed. Cellular interactions in development. New York, NY:IRL press, 93-127) by PCR amplification over two stages. Oligonucleotides (see "Mutagenesis primers" below) containing mismatched bases, together with 5'-agaacccactgcttactggct (SEQ ID NO: 14) and 3'-tgagcctgcttccagcagca (SEQ ID NO: 15) primers were used to generate two overlapping products. Following gel purification the two overlapping PCR products were annealed together before final amplification using the 5' and 3' primers. The final PCR product was restricted and ligated into pIg vector. All mutant clones were verified by sequence analysis. Mutant ICAM-4Fc proteins were expressed in COS-7 cells as described in Simmons DL (1993, *supra*), and purified from culture supernatant on protein A-Sepharose.

Table 1: Mutagenesis primers (all shown in 5'-3' orientation)

F18A	tca gtg ccc GCc tgg gtg cgc (SEQ ID NO: 16)
	gcg cac cca gGC ggg cac tga (SEQ ID NO: 17)

	W19A	gtg ccc ttc GCg gtg cgc atg (SEQ ID NO: 18)
		cat gcg cac cGC gaa ggg cac (SEQ ID NO: 19)
	V20T	ccc ttc tgg ACg cgc atg agc (SEQ ID NO: 20)
		gct cat gcg cGT cca gaa ggg (SEQ ID NO: 21)
	R92E	gga aaa aca GAA tgg gcc ac (SEQ ID NO: 22)
		gt ggc cca TTC tgt ttt tcc (SEQ ID NO: 23)
	A94L	aca cgc tgg CTc acc tcc agg (SEQ ID NO: 24)
		cct gga ggt gAG cca gcg tgt (SEQ ID NO: 25)
	T95V	cgc tgg gcc GTc tcc agg at (SEQ ID NO: 26)
		at cct gga gAC ggc cca gcg (SEQ ID NO: 27)
	S96A	tgg gcc acc Gcc agg atc acc (SEQ ID NO: 28)
		ggt gat cct ggC ggt ggc cca (SEQ ID NO: 29)
	R97E	gcc acc tcc GAg atc acc gc (SEQ ID NO: 30)
		gc ggt gat cTG gga ggt ggc (SEQ ID NO: 31)
	W66A	ggg ccg ggt GCg gtg tct ta (SEQ ID NO: 32)
		ta aga cac cGC acc cgg ccc (SEQ ID NO: 33)
	K118E	aag ggc agg Gaa tac act tt (SEQ ID NO: 34)
		aa agt gta ttC cct gcc ctt (SEQ ID NO: 35)
	N160A	gat ctg gcc GCc gtg acc ttg (SEQ ID NO: 36)
		caa ggt cac gGC ggc cag atc (SEQ ID NO: 37)
	T162V	gcc aac gtg GTc ttg acc ta (SEQ ID NO: 38)

ta ggt caa gAC cac gtt ggc (SEQ ID NO: 39)

Results and Discussion

ICAM-4 is predicted to have two immunoglobulin superfamily I-set domains, domain 1 being N-terminal of the membrane anchored domain 2. On a molecular model of ICAM-4 (Spring *et al.* 2001, *supra*, and see Fig 2), based on the crystal structure of ICAM-2, the VLA-4 and α_v integrin binding epitope on ICAM-4 consists of eight residues and is located in domain 1, in between the ABE and CFG faces (Fig 3). Three of the residues, F18, W19, and V20, are positioned on the A strand (Fig 3a), and five residues, R92, A94, T95, S96 and R97 are on the G strand (Fig 3b). Each of these residues was identified as important for binding on the basis of a decrease in binding of the singly mutated ICAM-4 and the α_v integrin ligand (see Fig 4 panels A through L). These residues identify an epitope on the ICAM-4 molecule that straddles the edges of both the ABE and CC'FG face of domain 1.

There is also a published LFA-1/Mac-1 binding site (Hermand *et al.*, 2000, *supra*) on ICAM-4 which is comprised of 8 residues, T91, R52, E151, T154, W93, L80, R97 and W77. On domain 1, T91, W93 and R97 are on the G strand (residues 90-100), W77 and L80 are on the F strand (residues 77-87) and R52 is on the C strand (residues 47-54). On domain 2 E151 and T154 are on the C'-E loop (residues 150-158). Of these residues, all comprise the Mac-1 binding site (Fig 6 view A) however, W93, L80, R97 and W77 only comprise the LFA-1 binding site (Fig 6 view B).

In total, the footprint domain of the present invention comprises a wider area than that covered by the epitope defined by the residues mutated herein (see Figure 4). It comprises these residues, the residues described by Hermand *et al.* (2000, *supra*) and amino-acids in the surrounding area. The footprint comprises residues on the A, C, G and F strand of domain 1 and extends down to the CE loop in domain 2 (see Fig 1, 2, 3, 5, and 6).

Two other residues are thought to be involved in the interaction between ICAM-4 and its integrin ligands; W66, located on the E strand (residues 65-75) of domain 1 and

K118, which is found on the B strand (residues 116-126) of domain 2 (Fig 3 view A and residues c and d, respectively and Fig 4 panels I and J respectively). These mutations also decrease the level of adhesion between ICAM-4 and HT1080 cells.

In addition, an N-glycosylation site comprising residues N160, V161 and T162 is believed to have a role in the binding of ICAM-4 and its ligands. This site is located at the top of the E strand (residues 160-170) of domain 2 (see Fig 5 views A and B; N160 is arrowed by a, and T162 is arrowed by b). Mutation of N160 or T162 leads to an elevated level of adhesion between ICAM-4 and HT1080 cells (Fig 4 panels K and L). Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed that the N160A and T162V mutants have increased electrophoretic mobility than native ICAM-4, which suggests that these two "super adhesive" mutations prevent the N-glycosylation of asparagine 160.

Areas thought to be important in ICAM-4 binding are shown in Figs 1, 3, 5 and 6.

Figs 7-18 show inhibition of HEL cell binding and HT1080 cell binding to human and murine ICAM-4Fc in the presence of blocking peptide sequences (peptides svpFWVrms (SEQ ID NO: 9), tRwATSRit (SEQ ID NO: 10) and aWssLahcl (SEQ ID NO: 11)) and a control peptide (rqgktlrgp; SEQ ID NO: 13).

Our findings suggest that contact between ICAM-4 and its integrin ligands involves a large extent of the surface of ICAM-4, with the epitope on domain 1 being a critical site in mediating this interaction. Integrin-mediated adhesion to ICAM-4 may play a role in the formation of erythroblastic islands in the bone marrow (during erythropoiesis) and in the abnormal adhesion of red cells to activated endothelium and other cellular elements in the vasculature and wider reticuloendothelial system in the diseases mention above.

Example 2

Peptide Inhibition of Erythroblast Adhesion to ICAM-4

In Example 1, we identified an area on ICAM-4 that is important in its adhesion to αV

integrins and using this information we designed blocking peptides corresponding to the sequences of the A, D, F and G strands of domain 1. These peptides have the sequences S(15)VPFWVRMS (SEQ ID NO: 9; on A strand), R(56)QGKTLRGP (SEQ ID NO: 13; on D strand), A(76)WSSLAHCL (SEQ ID NO: 11; on F Strand) and T(91)RWATSRIT (SEQ ID NO: 10; on G strand). We have shown that early erythroblasts bind to ICAM-4 in the presence of TS2/16, an activating β1 antibody (unpublished observations). The adhesion of HEL cells to ICAM-4 is mediated by the α4β1 integrin but not the α5β1 integrin (Spring *et al.*, 2001, *supra*). Erythroblasts express only two integrins at this stage in differentiation: α4β1 and α5β1 (unpublished observations). Therefore we hypothesise that erythroblasts adhere to ICAM-4 via α4β1, although we have not ruled out the fact that α5β1 may be involved in this interaction.

We have utilised the blocking ICAM-4 peptides (i.e., SEQ ID NOs: 9, 10, 11 and 13 – see Example 1) in order to inhibit the adhesion of day 4 erythroblasts to ICAM-4 (see Fig 19). Erythroblast cultures were initiated from CD34 positive cells purified from pooled buffy coat residues (obtained from the National Blood Service, Bristol, UK) and maintained as described in Southcott *et al.* (1999, Blood 93: 4425-4435). Cell adhesion assays were performed as described in Example 1 above. Immulon-4 96 well plates (Dynes Technologies, Billingshurst, UK) were coated with 1μg/well goat-antihuman-Fc (Sigma, Poole, UK) for 24 hours at 4°C, washed three times with PBS and coated with 0.25μg/well Fc fusion ICAM-4 protein (ICAM-4Fc) for 18 hours at 4°C before blocking with 0.4% BSA PBS for 2 hours at 22°C. Erythroblasts were labelled with 10μg/ml 2',7'-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein acetoxymethyl ester (Sigma, Poole, UK) in assay buffer (Iscoves modified Eagle medium, 2mM EGTA, 0.1% BSA, 10μg/ml Immune globulin intravenous (human) (Cutter Biological, Newbury, Berks, UK)) for 15 minutes at 37°C. Erythroblasts were washed with assay buffer containing 2mM Mn^{2+}. Erythroblasts, at $5X10^4$ cells per well, were added to the ICAM-4Fc-coated plates for 30 minutes at 37°C, prior to being cyclically read on a fluorescence microplate reader (excitation 485nm, emission 530nm) and washed in assay buffer. The percentage of bound cells was calculated after each wash. Peptide inhibition was performed by incubating the cells with 500μM peptide and 5μg/ml TS2/16 (beta 1 activating antibody (IBGRL) at 0°C for 15 minutes before their addition to the ICAM-4Fc coated plates.

The F and the G strand peptides (SEQ ID NOs: 10 and 11, respectively) inhibit adhesion whereas the strand A and D (SEQ ID NOs: 9 and 13, respectively) peptides had no effect. This suggests, along with the data already provided of the peptide inhibition of HEL cell – ICAM-4 adhesion (see Example 1), that the area of interaction with α4β1 on ICAM-4 lies in the F and G strands of domain 1. Therefore, the peptides of SEQ ID NOs: 9, 10 and 11 are useful tools allowing blocking of further ICAM-4 integrin interactions that are important in erythropoiesis and in the pathology of sickle cell disease, for example.

Example 3

Peptide and Antibody Inhibition of Neutrophil Adhesion to ICAM-4

ICAM-4 binds to platelet αIIbβ3 and the β2 integrins. These interactions may be part of the process whereby red cells participate in normal hemostatic processes and may also be relevant to thrombotic conditions such as deep vein thrombosis and vaso-occlusion in sickle cell disease. Indeed, it has recently been shown that during sickle cell crisis neutrophils that express β2 integrins, αLβ2 and αMβ2, bind not only inflamed endothelium but also adhere to erythrocytes. Since ICAM-4 is a likely, perhaps the only, candidate for mediating this erythrocyte adhesion with β2 integrins, we have assayed the *in vitro* adhesion of neutrophils to ICAM-4.

Utilising blocking β integrin subunit antibodies and our blocking ICAM-4 peptides (i.e., SEQ ID NOs: 9, 10, 11 and 13 – see Example 1) in a microplate neutrophil adhesion assay. Neutrophils were purified from buffy coats (obtained from the National Blood Service, Bristol, UK) as described in Henderson *et al.* (1987, Biochem. J. 246: 325-329). Cell adhesion assays were performed as described in Example 1 above. Immulon-4 96 well plates (Dynes Technologies, Billingshurst, UK) were coated with 1μg/well protein A (Sigma, Poole, UK) for 24 hours at 4°C, washed three times with PBS and coated with 0.125μg/well Fc fusion ICAM-4 protein (ICAM-4Fc) for 18 hours at 4°C before blocking with 0.4% BSA PBS for 2 hours at 22°C. Neutrophils were labelled with 10μg/ml 2',7'-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein acetoxymethyl

ester (Sigma, Poole, UK) in assay buffer (Iscoves modified Eagle medium, 2mM EGTA, 0.1% BSA for 15 minutes at 37°C. Neutrophils were washed with assay buffer containing 2mM Mn^{2+}. Neutrophils, at $5X10^5$ cells per well, were added to the ICAM-4Fc-coated plates for 10 minutes at 37°C, prior to being cyclically read on a fluorescence microplate reader (excitation 485nm, emission 530nm) and washed in assay buffer. The percentage of bound cells was calculated after each wash. Peptide and antibody inhibition was performed by incubating the cells with 500μM peptide and 25μg/ml antibody at 0°C for 15 minutes before their addition to the ICAM-4 Fc coated plates. Antibodies used were β_1 Mab13 (Yamada UK), β_2 TS1/18 (IBGRL) and β_3 PM6/13 (Serotec, UK).

We show in Figure 20 that neutrophil – ICAM-4 adhesion is mediated by β2 integrins (αLβ2 and αMβ2) and that it is likely to involve an interaction with the G and F strands of domain 1 of ICAM-4 as opposed to the A strand. These results are consistent with a previous site directed mutagenesis study of ICAM-4 that identified 8 residues T91, R52, E151, T154, W93, L80, R97 and W77 as important for adhesion to the β2 integrins (Hermand *et al.*, 2000, *supra*). All of these residues are involved in binding αMβ2 but only W93, L80, R97 and W77 comprise the αLβ2 binding site. T91, W93, L80, R97 and W77 are all located on the G and F strands of domain1 of ICAM-4.

Neutrophils bind the endothelium and to sickle red cells and thus are likely to be important in the blockage of capillaries (vaso-occlusion) in sickle cell disease. Example 3 shows that the adhesion between neutrophils and ICAM-4 is β_2 integrin mediated and that the peptides of SEQ ID NO: 10 and 11 inhibit this interaction. This means that antagonists to ICAM-4 such as SEQ ID NO: 10 and 11 could be used to affect (for example, inhibit) hemostatic processes as well as thrombotic conditions such as deep vein thrombosis and vaso-occlusion in sickle cell disease.

Sequence listing (partial)

SEQ ID NO: 1 - amino acid sequence of (mature) human ICAM-4 (See SWISSPROT accession No. Q14773 and Bailly *et al.*, 1994, *supra*.)

AQSPKGSPLA PSGTSVPFWV RMSPEFVAVQ PGKSVQLNCS NSCPQPQNSS
LRTPLRQGKT LRGPGWVSYQ LLDVRAWSSL AHCLVTCAGK TRWATSRITA
YKPPHSVILE PPVLKGRKYT LRCHVTQVFP VGYLVVTLRH GSRVIYSESL
ERFTGLDLAN VTLTYEFAAG PRDFWQPVIC HARLNLDGLV VRNSSAPITL
MLAWSPAPTA LASGSIAALV GILLTVGAAY LCKCLAMKSQ A

SEQ ID NO: 2 – PFWVRMSPEFV (Strand A of ICAM-4)

SEQ ID NO: 3 – KTRWATSRITA (Strand G of ICAM-4)

SEQ ID NO: 4 – WSSLAHCLVTC (Strand F of ICAM-4)

SEQ ID NO: 5 – NVTLTYEFAAG (Strand E of ICAM-4)

SEQ ID NO: 6 – GRKYTLRCHVT (Strand B of ICAM-4)

SEQ ID NO: 7 – QNSSLRTP (Strand C of ICAM-4)

SEQ ID NO: 8 – LERFTGLDL (CE Loop of ICAM-4)

SEQ ID NO: 9 - SVPFWVRMS (corresponding to residues 15 to 23 of ICAM-4)

SEQ ID NO: 10 – TRWATSRIT (corresponding to residues 91 to 99 of ICAM-4)

SEQ ID NO: 11 - AWSSLAHCL (corresponding to residues 76 to 84 of ICAM-4)

SEQ ID NO: 12 – Nucleotide sequence corresponding to mature human ICAM-4 (without signal peptide-encoding nucleotides)

GCGCAAAGCCCCAAGGGTAGCCCTCTCGCGCCCTCCGGGACCTCAGTGCCCTTCTGGGTG
101 160

CGCATGAGCCCGGAGTTCGTGGCTGTGCAGCCGGGGAAGTCAGTGCAGCTCAATTGCAGC
161 220

AACAGCTGTCCCCAGCCGCAGAATTCCAGCCTC**CGC**ACCCCGCTGCGGCAAGGCAAGACG
221 280

CTCAGAGGGCCGGGT**TGG**GTGTCTTACCAGCTGCTCGACGTGAGGGCC**TGG**AGCTCC**CTC**
281 340

GCGCACTGCCTCGTGACCTGCGCAGGAAAA**ACACGCTGGGCCACCTCCAGG**ATCACCGCC
341 400

TACAAACCGCCCCACAGCGTGATTTTGGAGCCTCCGGTCTTAAAGGGCAGG**AAA**TACACT
401 460

TTGCGCTGCCACGTGACGCAGGTGTTCCCGGTGGGCTACTTGGTGGTGACCCTGAGGCAT
461 520

GGAAGCCGGGTCATCTATTCCGAAAGCCTG**GAG**CGCTTC**ACC**GGCCTGGATCTGGCC***AAC***
521 580

GTG***ACC***TTGACCTACGAGTTTGCTGCTGGACCCCGCGACTTCTGGCAGCCCGTGATCTGC
581 640

CACGCGCGCCTCAATCTCGACGGCCTGGTGGTCCGCAACAGCTCGGCACCCATTACACTG
641 700

ATGCTCGCTTGGAGCCCCGCGCCCACAGCTTTGGCCTCCGGTTCCATCGCTGCCCTTGTA
701 760

GGGATCCTCCTCACTGTGGGCGCTGCGTACCTATGCAAGTGCCTAGCTATGAAGTCCCAG
761 820

GCG
821-823

Underlined and in bold are the nucleotides which encode residues defining the footprint domain (F18, W19, V20, R92, A94, T95, S96, R97, T91, R52, E151, T154, W93, L80, W77).

In bold and in italics are the nucleotides encoding the "super-adhesive" residues involved in the N-glycosylation site (N160 and T162).

In bold are the nucleotides encoding W66 and K118.

SEQ ID NO: 40 – Combined nucleotide and amino acid sequence of mature human ICAM-4

```
1                                                                  20
 A  Q  S  P  K  G  S  P  L  A  P  S  G  T  S  V  P  F  W  V
GCGCAAAGCCCCAAGGGTAGCCCTCTCGCGCCCTCCGGGACCTCAGTGCCCTTCTGGGTG
101                                                               160

21                                                                 40
 R  M  S  P  E  F  V  A  V  Q  P  G  K  S  V  Q  L  N  C  S
CGCATGAGCCCGGAGTTCGTGGCTGTGCAGCCGGGGAAGTCAGTGCAGCTCAATTGCAGC
161                                                               220

41                                                                 60
 N  S  C  P  Q  P  Q  N  S  S  L  R  T  P  L  R  Q  G  K  T
AACAGCTGTCCCCAGCCGCAGAATTCCAGCCTCCGCACCCCGCTGCGGCAAGGCAAGACG
221                                                               280

61                                                                 80
 L  R  G  P  G  W  V  S  Y  Q  L  L  D  V  R  A  W  S  S  L
CTCAGAGGGCCGGGTTGGGTGTCTTACCAGCTGCTCGACGTGAGGGCCTGGAGCTCCCTC
281                                                               340

81                                                                100
 A  H  C  L  V  T  C  A  G  K  T  R  W  A  T  S  R  I  T  A
GCGCACTGCCTCGTGACCTGCGCAGGAAAAACACGCTGGGCCACCTCCAGGATCACCGCC
341                                                               400

101                                                               120
 Y  K  P  P  H  S  V  I  L  E  P  P  V  L  K  G  R  K  Y  T
TACAAACCGCCCCACAGCGTGATTTTGGAGCCTCCGGTCTTAAAGGGCAGGAAATACACT
401                                                               460

121                                                               140
 L  R  C  H  V  T  Q  V  F  P  V  G  Y  L  V  V  T  L  R  H
TTGCGCTGCCACGTGACGCAGGTGTTCCCGGTGGGCTACTTGGTGGTGACCCTGAGGCAT
461                                                               520

141                                                               160
 G  S  R  V  I  Y  S  E  S  L  E  R  F  T  G  L  D  L  A  N
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GGAAGCCGGGTCATCTATTCCGAAAGCCTGGAGCGCTTCACCGGCCTGGATCTGGCCAAC
521 580

161 180
V T L T Y E F A A G P R D F W Q P V I C
GTGACCTTGACCTACGAGTTTGCTGCTGGACCCCGCGACTTCTGGCAGCCCGTGATCTGC
581 640

181 200
H A R L N L D G L V V R N S S A P I T L
CACGCGCGCCTCAATCTCGACGGCCTGGTGGTCCGCAACAGCTCGGCACCCATTACACTG
641 700

201 220
M L A W S P A P T A L A S G S I A A L V
ATGCTCGCTTGGAGCCCCGCGCCCACAGCTTTGGCCTCCGGTTCCATCGCTGCCCTTGTA
701 760

221 240
G I L L T V G A A Y L C K C L A M K S Q
GGGATCCTCCTCACTGTGGGCGCTGCGTACCTATGCAAGTGCCTAGCTATGAAGTCCCAG
761 820

241
A
GCG
821-823

Underlined and in bold are the mutated residues which comprise the footprint (F18, W19, V20, R92, A94, T95, S96, R97, T91, R52, E151, T154, W93, L80, W77).
In bold and in italics are the "super-adhesive" residues involved in the N-glycosylation site (N160 and T162).
W66 and K118 are shown in bold alone.